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Prediction of Therapy-Related Acute Myelogenous Leukemia (AML) and Myelodysplastic Syndrome (MDS) After Autologous Bone Marrow Transplant (ABMT) for Lymphoma

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Therapy-related acute myelogenous leukemia and myelodysplastic syndrome (t-AML/MDS) are being reported with increasing frequency as a complication of ABMT for Hodgkin's disease and non-Hodgkin's lymphoma. At present there is no method available to predict who is at risk or is destined to develop this nearly universally fatal disorder. We therefore investigated whether clonal growth of cells is predictive of the development of t-AML/MDS. In a patient who developed secondary AML/MDS 18 months after ABMT, X-linked clonality analysis at the human androgen receptor locus was performed on serial banked samples, and documented transition from polyclonal to clonal hematopoiesis. Clonal cells could be identified 6 months after transplant (1 year prior to the diagnosis of t-AML/MDS), at a time when there was no morphologic or clinical evidence of disease. Clonality analysis can be predictive of the development of t-AML/MDS after ABMT and may offer important insights into associated risk factors and strategies to minimize the risk of t-AML/MDS. Am. J. Hematol. 56:45–51, 1997. © 1997 Wiley-Liss, Inc.

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INTRODUCTION

ABMT has proven to be a significant advance in the treatment of relapsed, refractory, and poor prognosis Hodgkin's disease (HD) and non-Hodgkin's lymphoma (NHL), with cure rates approaching 40–50% in patient subgroups where expected survival was lower than 20% [1]. However, secondary neoplasia, principally myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML), has recently been recognized as a frequent, often fatal, late complication of intensive therapy [2–5]. The actual incidence of therapy-related AML/MDS (t-AML/MDS) has not been clearly defined but ranges from 6–10% with the actuarial risk reported as high as 18% at 6 years [2], which may limit the potential benefit of ABMT for HD and NHL.

X-linked clonality assays may offer a mechanism for identifying individuals at risk for the development of t-AML/MDS. One of the earliest abnormalities that one can define in an evolving neoplasm is clonal growth of cells, denoting acquisition of somatic mutation(s) that give selective growth advantage. X-linked clonality analysis offers several benefits over other methods of detecting clonal proliferation [6]; in particular, they do

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not rely on specific tumor markers or cytogenetic abnormalities and can thus determine clonality in any informative female.

In this report we use the human androgen receptor assay [6–11] (HUMARA; Fig. 1) for clonality determination in an informative female patient who developed t-AML/MDS after ABMT. We found clonal hematopoiesis to be predictive of the development of t-AML/MDS in this individual. Further investigation of this patient population using the HUMARA assay will determine the true predictive nature and utility of this analysis.

MATERIALS AND METHODS

DNA Isolation

Informed consent was obtained prior to utilization of all patient material. DNA was isolated from marrow samples as follows: PMN cells were separated from mononuclear cells by density gradient centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden). PMNs were isolated in the erythrocytic phase (greater than 95% purity by Wright stain) and subsequently lysed with Triton X-100 (Sigma, St. Louis, Mo) lysis solution (0.32 M Sucrose; 10 mM Tris pH 7.5; 5 mM $MgCl_2$; 1% Triton X-100). PMNs were digested with proteinase K in SDS buffer (Proteinase K 2 mg/ml, 5% SDS) at 37°C for 24 h, then DNA was extracted with phenol, phenol/chloroform, and chloroform, precipitated with 2 volumes of absolute ethanol and 1/10 volume of 3 M sodium acetate, and resuspended in Tris-EDTA (TE) buffer.

HUMARA Clonality Assay

A schema for the HUMARA clonality assay is shown in Figure 1. Briefly, DNA was digested with the methylation sensitive restriction endonuclease HpaII. HpaII will cleave unmethylated, active X alleles, precluding amplification of these alleles by PCR primers shown. In a polyclonal population of cells, both the maternal and paternal X alleles will be amplified and discriminated as two bands on a denaturing polyacrylamide gel by the variable CAG repeat. In contrast, for a clonal population of cells, only the maternal or the paternal allele will be amplified, giving a single band. The assay was carried out as previously described [7] with the following modifications, including use of a single-strand labeling method.

Kinasing primer protocol. Two microliters (5 pmol/μl) of primer HUMARA I was added to 10 × kinase buffer (1 μl, Boehringer Mannheim, Indianapolis, IN), γ-^{32}P ATP (6 μl, 3,000 μCi/mmol), polynucleotide kinase (0.6 μl, Boehringer Mannheim), and H_2O (0.8 μl), followed by incubation at 37°C for 30 min and inactivation of PK at 90°C for 2 min.

Pre-cutting of genomic DNA. Genomic DNA was pre-cut by mixing sample DNA (100 ng–1 μg in 2 μl) with *Hpa* II (1 μl, high concentration, 40 U/μl), *Rsa* I (0.5 μl, high concentration, 40 U/μl), L buffer (2 μl, Boehringer Mannheim), and H_2O (14.5 μl). An autocontrol was pre-cut in the same way except that *Hpa* II was omitted from the mix. Samples were incubated at

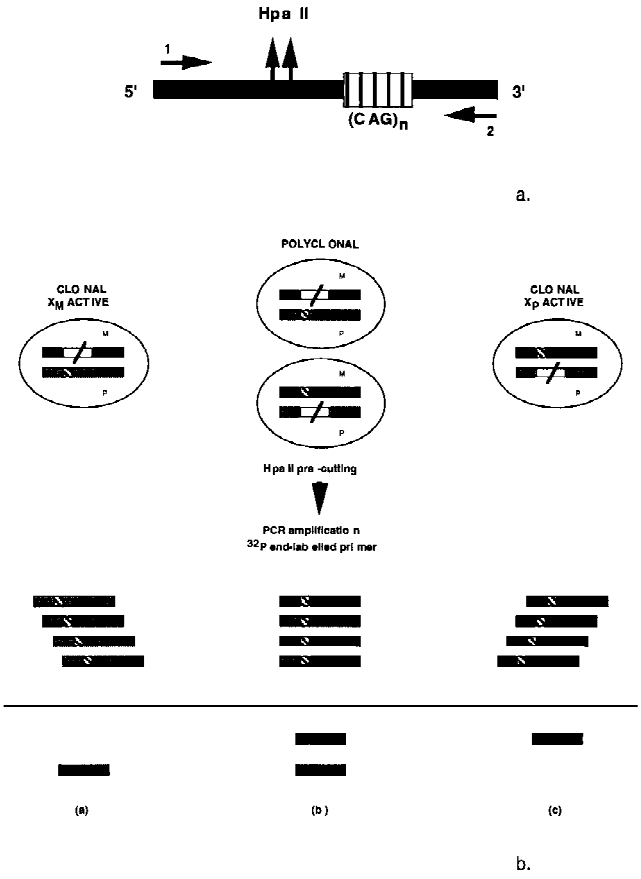


Fig. 1. a: HUMARA locus on chromosome X. The first exon of the human androgen receptor gene contains a highly polymorphic CAG repeat with more than 20 different alleles and a heterozygosity frequency of 90%. One hundred base pairs 5′ to the $(CAG)_n$ lies a site of differential methylation between Xa and Xi. The HUMARA assay has reliable methylation patterns which have been documented using an androgen receptor expression assay at the same locus [10]. Within the differential methylation site are 2 restriction sites for the methylation sensitive restriction endonuclease Hpa II. Hpa II will cleave unmethylated, active X alleles, precluding amplification of these alleles by PCR primers 1 and 2. b: The HUMARA clonality assay. DNA is digested with Hpa II and amplified by PCR using ^{32}p end-labeled primers. In a polyclonal population of cells (b), both the maternal and paternal X alleles will be amplified and discriminated as two bands of different molecular weight on a denaturing polyacrylamide gel by the variable CAG repeat. In contrast, for a clonal population of cells (a, c) where all cells are derived from a single common progenitor, only the maternal or paternal allele will be amplified, giving a single band. In accordance with published literature, patients are considered to have clonal hematopoiesis if the corrected allelic ratio (Cr) is > 3:1. Ratios between 1:1 and 3:1 (i.e., between 1 and 3) are considered to represent polyclonal hematopoiesis.

37°C overnight, and heat inactivated at 95°C for 10 min prior to amplification.

PCR amplification of the HUMARA locus. Two microliters of digested DNA was added to 23 μl of a PCR mix containing buffer (10×: 500 mM NaCl, 100 mM Tris-HCl, pH 8.2; 15 mM $MgCl_2$, 0.1% gelatin); dNTPs (200 μM each); primer HUMARA I: 5′-GCTGTGAAGGTTGCTGTTCCTCAT-3′, and primer HUMARA II: 5′-TCCAGAATCTGTTCCAGAGCGTGC-3′ (12.5 pmol each); DMSO (0.75 μl, Sigma); γ-^{32}P end labeled HUMARA I primer (1.25 pmol), Taq polymerase (0.5 U, Cetus, Norwalk, CT); H_2O to a final volume of 23 μl. Samples were amplified on a programmable thermal cycler (MJ Research, Inc., Cambridge, MA) with initial DNA denaturation at 94°C for 3 min, then 28 cycles starting with 94°C for 45 sec, 60°C for 30 sec, and 72°C for 30 sec. At the end of amplification 12.5 μl of formamide loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each sample, and samples were denatured at 95°C for 3 min and chilled rapidly. Amplified PCR products (8–10 μl) were electrophoresed on a 4% acrylamide-urea-formamide denaturing gel at 80 watts for 3.5 hr.

Quantitation of alleles. Dried gels were exposed to a phosphor screen for 24 hr and scanned on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The ratio between the two X-linked alleles was quantitated using Image Quant software. The allele ratio is the ratio between the two X-linked alleles in a given sample. The corrected ratio (Cr) is the allele ratio of the pre-cut sample divided by the allele ratio of the non-precut sample of the same specimen. In accordance with published literature, patients are considered to have clonal hematopoiesis if the Cr is >3:1 [12]. Ratios between 1:1 and 3:1 are indicative of polyclonal hematopoiesis. The percentage of clonal cells/sample was determined from the Cr according to the theoretical curve generated by equation: $| X/X + 1 - [1 - (X/X + 1)] | = y$ where $X =$ Cr, $Y =$ percentage of clonal cells, $X/X + 1 =$ the percentage of cells in the sample which contain the top allele, and $1 - (X/X + 1) =$ the percentage of cells in the sample which contain the bottom allele. This equation can be simplified to $| X - 1/X + 1 | = Y$.

Cytogenetic Analysis

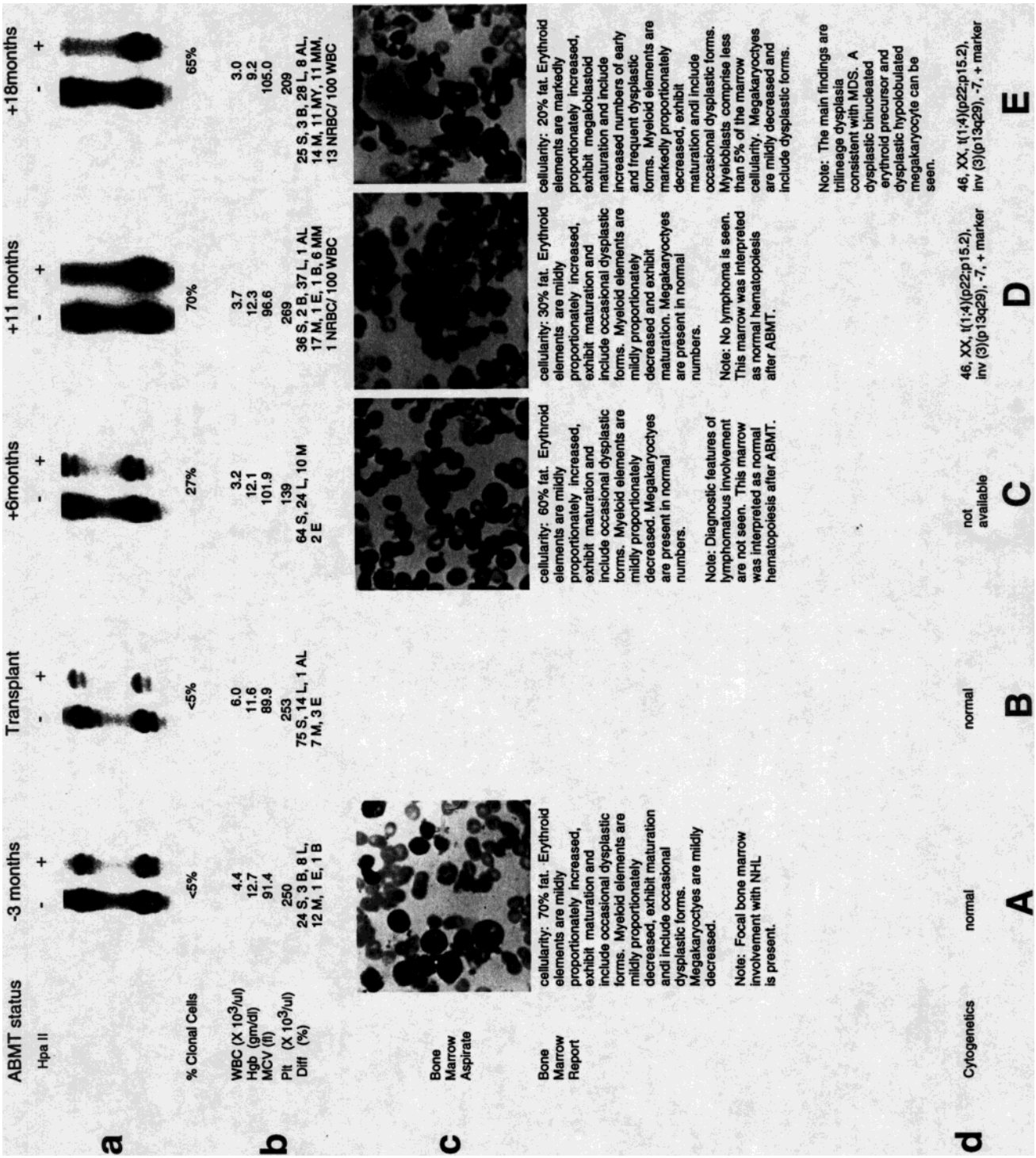
Cytogenetic analyses were performed from 24-hr cultures of bone marrow aspirates. Cells were grown at 37°C in 5% CO_2 in RPMI 1640 with 16% fetal bovine serum, 2 mmol/L L-glutamine, and antibiotics. After a brief exposure to colcemid (10 μg/ml), cells were pelleted and the cell pellets were resuspended in 0.075 M KCl solution. After 20 min, cells were pelleted, and after discarding the hypotonic KCl solution, cells were fixed in freshly prepared 3:1 methanol/acetic acid for 24 hr. After 2 changes of fixatives, cells were suspended in 1 ml of fresh fixative. Aliquots of cell suspension were dropped on cold, wet slides and allowed to air dry. Slides were stained in 30 μg/ml quinicrine mustard solution and mounted in pH 5.8 tris-maleate buffer. Well-banded metaphase spreads were photographed using a Leitz (Wetzlar, Germany) aristoplan fluorescence microscope.

RESULTS

Patient C.M. was well until June, 1990 when she presented with left cervical adenopathy. After no response to antibiotics, a lymph node biopsy was performed, revealing follicular small cleaved cell lymphoma. Further staging revealed diffuse lymphadenopathy and bone marrow involvement. Cytogenetics were normal at the time of diagnosis but the Bcl 2-Ig heavy chain gene rearrangement was identified by PCR. After an incomplete response to two cycles of CVP (cytoxan, vincristine, prednisone) and five cycles of CHOP (cytoxan, adriamycin, vincristine, prednisone) chemotherapy, characterized by persistent disease in her marrow and persistent evidence of the rearrangement by PCR, she received cytoxan/TBI as myeloablative therapy and underwent ABMT [13]. Post-transplant she had an uneventful course, remaining in remission with negative PCR. However, she had progressive refractory anemia and leukopenia and was diagnosed with MDS 18 months after transplant. At the time of diagnosis of t-MDS, physical exam was essentially unremarkable. She subsequently died of complications after an allogeneic bone marrow transplant, approximately 5 years after initial presentation.

Clonality analysis of patient C.M. DNA from bone marrow using the HUMARA clonality assay 3 months prior to and at the time of transplant showed polyclonal hematopoiesis (Fig. 2a, A and B). Cytogenetics 3 months before and at the time of transplant were normal. Six months after transplant and 1 year prior to the diagnosis of MDS, a clonal population of cells was detected in bone marrow (27% clonal cells quantitated by phosphorimaging analysis; Fig. 2a, C). At this time the patient's bone marrow aspirate and biopsy showed no evidence of myelodysplastic syndrome. Peripheral blood revealed a white blood count of 3.2×10^3 cells/μl (64% polymorphonuclear leukocytes, 24% lymphocytes, 10% monocytes, and 2% eosinophils), hemoglobin 12.1 (MCV 101.9), platelets 139×10^3/μl. Cytogenetics were not performed on this sample. Clonality analysis of bone marrow at 11 months after transplant revealed a further clonal shift, with the majority of cells clonally derived (Fig. 2a, D). Again, bone marrow examination revealed no evidence of t-AML/MDS. The minimal amount of dysplasia noted on this and prior biopsies (Fig. 2c, A and C) was considered indicative of normal hematopoiesis after intensive chemotherapy. Peripheral blood revealed

a white blood count of 3.7×10^3 cells/μl (36% polymorphonuclear leukocytes, 2% bands, 37% lymphocytes, 1% atypical lymphocytes, 17% monocytes, 1% eosinophils, 1% basophils, and 6 metamyelocytes with 1 nucleated red blood cells/100 white blood cells), hemoglobin 12.3 (MCV 96), platelets 269×10^3/μl. Cytogenetics were not performed at this time. MDS was diagnosed 18 months post-transplant with bone marrow showing trilineage dysplasia (Fig. 2c, E). There was no fibrosis in the marrow and less than 5% myeloblasts. Peripheral blood at this time revealed a white blood count of 3.0×10^3 cells/μl (25% polymorphonuclear leukocytes, 3% bands, 28% lymphocytes, 8% atypical lymphocytes, 10% monocytes, 11% myelocytes, 11% metamyelocytes with 13 nucleated red blood cells/100 white blood cells), hemoglobin 9.2 (MCV 105), platelets 209×10^3/μl. Karyotype analy-



sis revealed a translocation between the long arms of chromosome Nos. 1 and 4, a pericentric inversion of chromosome No. 3, monosomy of chromosome No. 7 and a marker chromosome (smaller than a G group size chromosome) in sixteen of eighteen metaphases analyzed from 24-hr cultures. In addition, one cell showed a deletion in the long arm of chromosome 6. The remaining two metaphases contained a normal, diploid female karyotype. Clonality analysis of this sample showed persistent clonal hematopoiesis (Fig. 2a, E). Cytogenetic analysis was performed on banked bone marrow cells from aspirates obtained at transplant, 6 and 11 months post-transplant. While no well-banded metaphases were obtained from the sample obtained 6 months post-transplant, 24/24 cells at the time of transplant demonstrated a normal female karyotype and 11/11 cells from the 11-month post-transplant sample demonstrated the abnormal clone.

DISCUSSION

ABMT has proven effective in curing a significant subgroup of patients with HD and NHL who would otherwise be destined to die of their disease. However, the actual incidence of therapy-related AML/MDS (t-AML/MDS), which is frequently a fatal complication, probably ranges from 6–10% in this population with the actuarial risk reported as high as 18% at 6 years [2–5]. It is, therefore, imperative that methods be developed both to ascertain the basis for the increased risk of AML/MDS and to minimize this risk in patients undergoing ABMT. We have demonstrated the evolution of clonal hematopoiesis in a patient shortly after transplant and 1 year prior to the diagnosis of t-AML/MDS, at a time when there was no clinical or morphologic evidence of secondary malignancy. The HUMARA assay thus anticipated the development of MDS.

T-AML/MDS in this setting could be due solely to pretransplant therapy. In this model, alkylating agents and/or traditional therapy would induce genetic damage predisposing to the development of MDS. Bone marrow harvested from these patients is then reinfused after the transplant-conditioning regimen, eventually leading to the MDS phenotype. Several lines of evidence support this model: (1) the development of secondary AML/MDS in the nontransplant setting correlates with the amount of alkylating agent received [14], and patients undergoing transplant have often received multiple chemotherapeutic regimens incorporating alkylators for relapsed disease in addition to their original induction regimen; (2) the long interval between initial therapy and the development of t-AML/MDS correlates closely with that seen for patients receiving alkylating agent therapy who develop t-AML/MDS in the non-transplant setting [2]; and (3) preliminary data from our laboratory suggests that a significant proportion of NHL patients have clonal hematopoiesis at the time of transplant [9].

Alternatively, AML/MDS after ABMT may be due to endogenous hematopoietic progenitors, which survive the intensive transplant-conditioning regimen. In particular, concern has been raised that total body irradiation (TBI) in the conditioning regimen may contribute to development of AML/MDS. However, of 9 patients with MDS after ABMT reported by Miller et al., 4 had transplant conditioning using TBI, while 5 had chemotherapy alone for conditioning [5]. This model may be somewhat less likely since AML/MDS rarely occurs after allogeneic BMT where similar conditioning regimens are used, although the allogeneic BMT graft vs. leukemia effect would alleviate the risk of AML/MDS. If X-linked

Fig. 2. Evolution of clonal hematopoiesis after autologous bone marrow transplant (ABMT). a: X-linked clonality analysis of serial bone marrow samples obtained from patient C.M. using the HUMARA assay. DNA was digested, amplified with primers that flank the CAG repeat, and run on PAGE. Dried gels were exposed to a phosphor screen for 24 hr and scanned on a PhophorImager (Molecular Dynamics). Allele ratios were determined using Imagequant software. The percentage of clonal cells/sample was determined from the clonality ratio according to the theoretical curve generated by equation: $|(x - 1)/(x + 1)| = y$, where x = clonality ratio and y = % clonal cells. The percentage of clonal cells in a highly clonal population of cells can be underestimated due to background. ''+'' denotes DNA precut with Hpa II; ''–'' denotes DNA not precut with Hpa II. Non-precut DNA samples show the two X-linked alleles corresponding to the paternal and maternal copies of the androgen receptor locus. Shadow bands below the major band are characteristic of amplified repeat sequences. Precut DNA from samples A and B gives two bands of the same relative intensity reflecting a polyclonal population of cells (>90%). Precut DNA from samples C–E show progressive dominance of the lower allele, reflecting clonal derivation of cells in these samples. The amplified allele corresponds to the methylated inactive X allele. b: Peripheral blood counts obtained on the day of DNA procurement. S = polymorphonuclear leukocyte; B = band form; L = lymphocyte; AL = atypical lymphocyte; M = monocyte; E = eosinophil; B = basophil; MM = metamyelocyte; MY = myelocyte; NRBC = nucleated red blood cell; WBC = white blood cell; Hgb = hemoglobin; MCV = mean cell volume; Plt = platelet; Diff = differential. c: Serial bone marrow aspirates (40×) showing evolution of myelodysplasia. A and C show hematopoiesis without significant dysplasia 3 months prior to transplant and 6 months after transplant, respectively. D demonstrates occasional dysplastic erythroid precursors, however, the diagnosis of myelodysplasia was not entertained. E demonstrates trilineage dysplasia consistent with the diagnosis of myelodysplasia. d: Cytogenetics performed on bone marrow specimens document the acquisition of karyotypic abnormalities associated with the diagnosis of MDS.

clonality assays are predictive of development of secondary AML/MDS, then the time to development of clonality in relation to transplant should allow for delineation of the risk factors involved. It is important to distinguish the relative contributions of these two possibilities to determine approaches that would prevent t-AML/MDS.

For example, if pretransplant chemotherapy and/or radiation therapy were the primary risk factors for development of secondary AML/MDS, then marrow harvest and cryopreservation could be considered shortly after the time of diagnosis for patients presenting with poor prognostic factors [15] who are more likely to require intensive chemotherapy, including ABMT. Early harvest could serve as a source of host stem cells without prolonged exposure to the mutagenic effects of alkylating agents and radiation therapy. As well, patients with clonal hematopoiesis prior to transplant may be candidates for allogeneic bone marrow transplant or ABMT using purified, normal, polyclonal progenitors (e.g., CD34+ cells) [16]. Finally, we (unpublished data) and others [17] have documented the persistence of polyclonal cells in some patients with MDS, suggesting that purification of normal, polyclonal progenitors may be possible even after expression of the malignant phenotype. These data may also be relevant in breast cancer, where there has been an increased risk of secondary AML/MDS associated with high-dose alkylating agent therapy. For example, eight cases of AML/MDS were recently reported in the NSABP B-25 trial, evaluating adjuvant high-dose cytoxan and adriamycin in 2,548 node positive breast cancer patients [18]. Based on this data, a reporting and monitoring plan is now in place for cases of secondary AML/MDS in all NCI-sponsored trials, and consent forms include the risk of secondary AML/MDS associated with high-dose alkylating agent therapy. Prospective analysis of additional patients using X-linked clonality analysis will be necessary to address these possibilities.

One potential limitation of X-linked clonality assays is *extreme Lyonization*, which can mimic clonal derivation of cells in some patients. According to Lyon's hypothesis [19], all X chromosomes in a cell in excess of one are inactivated on a random basis during early embryogenesis. In each cell, the inactivation process is unrelated to the same process occurring in other cells, so that the Lyonization ratio (Xp active/Xm active) in the population follows a binomial distribution in which extreme Lyonization is a statistically rare event. According to this principle of random X inactivation in normal females, 50% of cells should contain Xp in the active state and 50% of cells should contain Xm in the active state. However, recent studies have suggested that severe Lyonization occurs at a substantially higher frequency than originally thought. Gale et al. found significant unequal Lyonization in 23% of normal females using PGK and HPRT probes [20]. For this reason, lineage specific cells should be analyzed for each female to evaluate the possibility of extreme Lyonization. While analysis of T-cells in our patient demonstrated polyclonal hematopoiesis (data not shown), it should be noted that this patient serves as her own internal control, as analysis of her bone marrow over time demonstrates the evolution of clonal hematopoiesis from a bone marrow that initially demonstrated unequivocal polyclonal hematopoiesis.

It will be important to correlate clonality data with karyotype analysis to establish the relationship between selective growth advantage, demonstrated by clonal hematopoiesis, and discernible cytogenetic aberrations associated with progression of clonal hematopoiesis to the malignant phenotype. While cytogenetics were normal 3 months prior to transplant, karyotype analysis was not performed on subsequent samples, prior to the diagnosis of t-AML/MDS. Retrospective analysis of stored bone marrow 11 months after transplant revealed the abnormal clone. It is, therefore, possible that there were cytogenetic abnormalities at the time when clonal hematopoiesis was first detected by the HUMARA assay. One other patient with t-AML after ABMT for relapsed Hodgkin's disease has been reported to have clonal hematopoiesis by X-linked clonality analysis at the PGK locus 7 months prior to the diagnosis of secondary malignancy [21]. Cytogenetics were not available on this patient.

In summary, we have documented that clonality analysis, using the X-inactivation based HUMARA clonality assay, can be predictive of the development of t-AML/MDS after ABMT for lymphoma. Prospective analysis of a large number of patients will be required to validate these findings.

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